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<p>(21) International Application Number: PCT/US97/02935 (22) International Filing Date: 20 February 1997 (20.02.97)</p> <p>(30) Priority Data: 60/012,071 22 February 1996 (22.02.96) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 60/012,071 (CIP) Filed on 22 February 1996 (22.02.96)</p> <p>(71) Applicant (for all designated States except US): WIVORX PHARMACEUTICALS, INC. [US/US]; 2nd floor, 3212 Nebraska Avenue, Santa Monica, CA 90404 (US).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): SOON-SHIONG, Patrick [US/US]; 11755 Chenault Street, Los Angeles, CA 90049 (US).</p> <p>(74) Agent: REITTER, Stephen, E.; Gray, Cary Ware & Freidenrich, Suite 1600, 4365 Executive Drive, San Diego, CA 92121 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p><i>O 1 too broad O 2 no imports Note search (x)</i></p>
<p>(54) Title: METHOD FOR GROWING PLANT CELLS IN LIQUID SUSPENSION CULTURES, AND CHEMOTHERAPEUTIC AGENTS DERIVED FROM PLANTS</p> <p>(57) Abstract</p> <p>A microgravity liquid-suspension culture method has been developed for growing aggregates of plant cells which produce naturally occurring chemical products for use as therapeutic agents, including chemotherapy for cancer and other diseases. The invention method involves the use of a complex cell-culture medium containing necessary nutrients and growth factors, a microgravity culture vessel for promoting three-dimensional growth, and a set of growth-promoting plant growth factors.</p>		

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METHOD FOR GROWING PLANT CELLS IN LIQUID SUSPENSION CULTURES, AND
CHEMOTHERAPEUTIC AGENTS DERIVED FROM PLANTS

FIELD OF THE INVENTION

The present invention relates to methods and compositions for the growth of plant cells and the production of naturally occurring therapeutic agents 5 including taxol, taxanes, homoharringtonine, antibiotics, immunosuppressives, antifungals, and antivirals.

BACKGROUND OF THE INVENTION

Higher plants are important sources of pharmaceuticals and specialty chemicals. Many of these 10 chemicals are secondary metabolites (not essential for plant growth) which protect the plant from various pathogens. Plant-derived pharmaceuticals are usually obtained by extraction of harvested plant materials or by chemical synthesis. Production of plant-derived 15 pharmaceuticals from liquid suspension culture is an enabling technology which could greatly increase the supply of chemotherapeutic chemicals such as taxol. Cell culture ultimately offers reliable year-round production of these valuable chemicals under controlled conditions. In 20 addition, it is expected that these methods can be continually improved in light of advances in technology and genetics. Cell culture ensures a limitless and continuous source of product and is not subject to weather or blight. Cell cultures can be induced to overproduce the product of 25 interest, thereby simplifying the process of separation and purification.

Free-living microorganisms (e.g., bacteria or fungi) possess tough cell walls for resistance to most environmental stresses. Microorganisms synthesize nearly

all of the biomolecules essential to life. However, cells from higher organisms (including plants) are structurally delicate and require constant supplements of specific nutrients to maintain viability. Large-scale processes are 5 better developed and less difficult for culturing bacteria than for culturing plant cells. Bacterial cells can be grown under vigorous agitation in large volumes of simple liquid medium.

In contrast, however, plant cells are more 10 difficult to grow as they are easily damaged by the shear stresses of turbulent fluid flow. In addition, plant cells require complex nutrient media to support cell growth. Plant cell cultures are established by isolating from the intact plant a living explant tissue, usually in the form 15 of a callus (which is an unorganized proliferating mass of undifferentiated plant cells). Induction of the callus requires an environment allowing some of the cells to divide and proliferate, provided by a special nutrient medium supplemented with plant growth regulators. Two 20 media (Gamborg B5 or Murashige & Skoog) have been widely used in a number of plant culture systems, although Gamborg B5 (with supplements) has been most effective in taxol-producing cultures.

Plant cell suspension cultures are induced from 25 established callus cultures by transferring callus into a type of liquid medium which favors suspension-culture growth. Plant cells typically grow as aggregates because of the failure of dividing cells to separate completely. Suspension cultures are advantageous because they are 30 easily scaled up for large scale (75,000 liter) mass production. Suspension cultures are the method of choice when designing bioreactor systems for production of desirable plant-derived products, since they are relatively homogeneous and carefully controlled.

Taxol is a diterpenoid alkaloid originally isolated from the bark of the pacific yew, *Taxus brevifolia*. The National Cancer Institute has shown that crude bark extracts exhibit anti-tumor activities, and 5 clinical trials have confirmed that taxol is extremely effective against various cancers (including ovarian and breast). Unfortunately, the supply of natural taxol is severely limited; several pacific yew trees are required to obtain enough of the drug to treat one patient, and the yew 10 has become nearly extinct due to logging. Although cultures of herbaceous plants have been relatively easy to grow, cultures of woody plants or conifers have usually been more difficult.

SUMMARY OF THE INVENTION

15 In accordance with the present invention, methods have been developed for the rapid and efficient initiation of cultures from different plants, e.g., different *Taxus* species, for their successful growth on artificial nutrient media, and for the production of high levels of useful 20 plant derived products, e.g., taxol and taxanes, in a short period of time.

The invention employs a microgravity culture vessel which provides a quiescent low-shear environment for the maintenance of plant cells in the form of high-fidelity 25 three-dimensional structures. The invention utilizes processes for growing plant cells under optimized conditions which lead to the production of large amounts of useful therapeutic agents. The invention encompasses the construction and use of improved or altered plant cells 30 which are especially suited to growth and production in suspension culture, including cells obtained by selective breeding or recombinant DNA methods. The invention extends to all therapeutic agents produced or manufactured by this process, including natural products as well as recombinant

products. The invention employs a complex cell-culture medium containing various nutrients and growth factors which are necessary or sufficient to promote long-term cell growth or multiplication and to avoid senescence or loss of 5 biological function. The invention involves the use of appropriate plant growth regulating factors to maintain plant cells in liquid suspension culture.

DETAILED DESCRIPTION OF THE INVENTION

10 In accordance with the present invention, there are provided methods for growing plant cells, said methods comprising cultivating, in nutrient medium, plant cells derived from callus culture or suspension culture or both.

15 As employed herein, "callus" is an unorganized proliferating mass of undifferentiated plant cells.

As employed herein "suspension culture" means structurally undifferentiated cells in various stages of aggregation dispersed throughout a liquid nutrient medium.

20 As employed herein "nutrient medium" refers to a medium suitable for cultivation of plant cell callus and suspension cultures, and may be either a growth medium or a production medium.

25 As employed herein, "growth medium" refers to a nutrient medium which promotes rapid growth of cultured cells.

As employed herein, "production medium" refers to a nutrient medium which promotes biosynthesis of a desired product (e.g., taxol) in cultured cells. As employed herein, "taxol" refers to a diterpenoid alkaloid originally

isolated from the bark of the pacific yew, *Taxus brevifolia*, and it possesses antitumor activity. As employed herein, "taxanes" refers to chemical compounds structurally related to taxol.

5 In a preferred aspect of the invention, growth of plant cells occurs within a vessel generating a quiescent microgravity environment to diminish shear stresses and to maintain three-dimensional cell-aggregate structures.

Products contemplated for production according to 10 the present invention include naturally occurring or genetically recombinant products useful as chemotherapeutic agents. Exemplary products include taxol, taxane or other compounds structurally similar to taxol, homoharringtonine, 15 antibiotic compounds, immunosuppressive compounds, antifungal compounds, antiviral compounds, and the like.

Taxol is present in many parts of the *Taxus* plant, but is most highly concentrated in its bark and new-growth needles. Explants from the bark or needles of *T. brevifolia* or *T. chinensis* have been successfully cultured 20 to produce taxol and related taxanes from both callus and suspension cultures. As used herein, "explants" are isolated samples of living plant tissue which have been separated from the intact plant.

For the preparation of *Taxus* cell lines, needles 25 and meristematic regions of the *Taxus* plant are removed and surface sterilized (e.g., using chlorine bleach) prior to introduction into the culture medium. Antimicrobial agents (e.g., phosphomycin, gentamycin sulfate, ampicillin, cefoxitin, or benlate, cloxacillin) may also be employed 30 for surface sterilization of plant material.

For callus and suspension growth according to the invention, media appropriate to the individual cell line

are used for induction and proliferation of the callus. As used herein, "proliferation" means an increase in total cell number. Gamborg B5 medium at pH 5.5 (between 3.0 and 7.0, but preferably between 4.0 and 6.0), supplemented with 5 0.2% casamino acids and 1-2 mg/L 2,4-dichlorophenoxyacetic acid is optimal for growth and subculture of callus. This medium contains an effective cell-growth-promoting concentration of water, sodium (Na^+) ions, potassium (K^+) ions, calcium (Ca^{++}) ions, magnesium (Mg^{++}) ions, zinc (Zn^{++}) 10 ions, chloride (Cl^-) ions, sulfate (SO_4^{2-}) ions, all essential amino acids, water-soluble vitamins, coenzymes, and glucose. Antibiotics (such as benlate or ceftoxin) are added to the medium to reduce explant losses due to microbial contamination. Elevated levels of carbohydrate 15 (e.g. 7% to 10% sucrose) have been employed to raise osmotic pressure and inhibit cell growth in a mature culture, thereby stimulating the accumulation of secondary metabolites such as taxol. Addition of other elicitors such as inexpensive salts (e.g. 20 to 50 mM sodium chloride 20 or potassium chloride) can likewise elicit secondary metabolite production. As used herein, "elicitors" are compounds added to the culture medium which stimulate secondary metabolite production. They can be abiotic (e.g., salts and sugars and metal ions) or biotic (e.g., 25 autoclaved fungi and bacteria).

Air is introduced into liquid cultures by means of tubing into the culture vessels. The temperature is typically maintained between 20°C and 26°C. Although cultures may be grown under any conditions of illumination, 30 when taxol-producing cells are being cultured, total taxol production is highest in cultures exposed to light (between 100 and 3000 foot candles).

Nutrients and factors supplemented into growth medium are critical for the long-term viability and health 35 of plant cells in culture. The culture medium is prepared

using a standard medium as a basis. Gamborg B5 medium at pH 5.5, supplemented with 0.2% casamino acids and 1-2 mg/L 2,4-dichlorophenoxyacetic acid is optimal for growth and subculture of callus. This medium contains effective cell-
5 growth-promoting concentrations of water, calcium ions, sodium ions, all essential amino acids, water-soluble vitamins, coenzymes, and glucose. Antibiotics (such as benlate or ceftoxin) are added to the medium to reduce explant losses due to microbial contamination. Elevated
10 levels of carbohydrate (e.g., 7% to 10% sucrose) have been employed to raise osmotic pressure and inhibit cell growth in a mature culture, thereby stimulating the accumulation of secondary metabolites such as taxol. Addition of other elicitors such as inexpensive salts (e.g., 20 to 50 mM
15 sodium chloride or potassium chloride) can likewise elicit secondary metabolite production

The suspension cultures are maintained for 1 to 8 weeks after subculturing, and are then harvested by removal and filtration of the growth medium employing
20 standard techniques. After the culture has been weighed, lyophilized, and ground to a fine powder, the desired product (e.g., taxol) can be recovered by suitable means, e.g., by extraction using conventional solvent extraction methods.

25 The microgravitational process employed in the practice of the present invention greatly enhances the ability to form and maintain three-dimensional living tissues. It simultaneously minimizes the fluid shear stress, provides three-dimensional freedom for cell and
30 substrate spatial orientation, and increases localization of cells, tissues, and substrates in a similar spatial region for significant periods during the cell culture. Thus, in the microgravity vessel employed in the practice of the present invention, cells, tissues, and substrates
35 rotate about an axis nearly perpendicular to gravity.

Particles of greatly different sedimentation rates orbit in particular paths and remain spatially localized for many minutes or hours. This allows particles sufficient interaction time to form multicellular structures and to 5 associate with each other.

Suitable vessel diameters are chosen based on the volume required for the intended quantity of cultured material, and which will allow a sufficient seeding density of cells, tissues, and substrates. As understood by those 10 of skill in the art, the outward particle drift due to centrifugal force is exaggerated at higher vessel radii and for rapidly sedimenting particles. Selected levels of shear stress may be introduced into the culture environment by differential rotation of the vessel components, as a 15 means for controlling the rate and size of tissue formation and for maintaining optimal particle sizes and associated sedimentation rates.

In accordance with another aspect of the present invention, there are provided plant cells, cultures, and 20 cell lines developed specifically for growth and production using the cell culture methods described herein.

In accordance with yet another aspect of the present invention, there are provided plant cells, cultures, and cell lines produced using the cell culture 25 methods described herein.

In accordance with yet another aspect of the present invention, there are provided therapeutic agents, both naturally occurring and genetically recombinant, which are produced using the cell culture methods described 30 herein.

The invention will now be described in greater detail by reference to the following non-limiting example.

EXAMPLE

Taxol-producing plant cells, when cultured under microgravity conditions, are stabilized with respect to the formation and maintenance of three-dimensional aggregates.

5. Thus, callus was introduced into a microgravity vessel containing culture medium and growth factors. Simulated microgravity was created (in ordinary unit gravity) by modulating the horizontal rotation of a culture vessel completely filled with culture medium containing the
10 matrix. These conditions cause cells to collocate in one spatial region and encourage the maintenance of aggregates because shear stresses arising from the relative motion of the medium with respect to the walls of the vessel are minimized.

15. While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

That which is claimed is:

1. A method for growing plant cells, said method comprising cultivating, in nutrient medium, plant cells derived from callus culture or suspension culture or both.

2. A method according to claim 1 wherein growth of said plant cells occurs within a vessel generating a quiescent microgravity environment to diminish shear stresses and to maintain three-dimensional cell-aggregate structures.

3. A method according to claim 1 wherein said plant cells produce a naturally occurring or genetically recombinant product for use as a chemotherapeutic agent.

4. A method according to claim 1 wherein said plant cells produce taxol.

5. A method according to claim 1 wherein said plant cells produce any taxane or other compound structurally similar to taxol.

6. A method according to claim 1 wherein said plant cells produce homoharringtonine.

7. A method according to claim 1 wherein said plant cells produce an antibiotic compound.

8. A method according to claim 1 wherein said plant cells produce an immunosuppressive compound.

9. A method according to claim 1 wherein said plant cells produce an antifungal compound.

10. A method according to claim 1 wherein said plant cells produce an antiviral compound.

11. Plant cells, cultures, and cell lines developed specifically for growth and production using the method of claim 1.

12. Plant cells, cultures, and cell lines produced by the method of claim 1.

13. Therapeutic agents, both naturally occurring and genetically recombinant, which are produced using the method of claim 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02935

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 5/02
US CL : 435/410, 420

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/420

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, AGRICOLA, BIOSIS
search terms: plant#, suspen?, culture#, suspension culture, nutrient#, hydroponic#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,312,740 A (SAITO et al.) 17 May 1994, see entire document.	1, 3-5, 11-13
Y		6-10
Y	US 5,030,573 A (PETIARD et al.) 09 July 1991, see entire document, especially column 7, line 39 through column 8, line 68.	1-13
X	US 5,409,828 A (FRENKEL et al.) 25 April 1995, see entire document, especially column 4, lines 57-66.	1, 11-13
Y		3-10

 Further documents are listed in the continuation of Box C. See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biosys abstract, abstract number 87:341533, SCHNEIDER et al. Metabolism of 2-2 4 Dichlorophenoxyisobutyric Acid and its Glucosyl Ester in Plants and Suspension Cultured Cells of Agrostemma-Githago. 1987. J. Plant Physiol. Vol 127 No. 1-2, pages 147-152, see Abstract.	1, 3-13
X	Biosys abstract, Abstract number 80:185463, BAGRATHISHVILI et al. Production of a Tea Plant Suspension Cell Culture. 1979. Fiziol. Rast. (MOSC) Vol 26, No. 2, pages 449-451, see Abstract.	1, 11-13
X,P ----	Biosys abstract, Abstract number 96:467066, PESTCHANKER et al. Kinetics of Taxol Production and Nutrient Use in Suspension Cultures of Taxus Cupidata in Shake Flasks and a Wilson-Type Bioreactor. Enzyme and Microbial Technol. 1996. Vol 19, No. 4, pages 256-260, see Abstract.	1, 3-5, 11-13 -----
Y,P		2, 6-10